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13. ABSTRACT *(Maximum 200 words)*

In analogy to the evolutionary processes that have brought natural enzymes to peak efficiency, we are developing molecular selection processes in the laboratory for preparing and improving antibodies possessing catalytic activity (abzymes). Over the three-year course of this project, we have achieved several important goals. We have exploited phage display methods for the first time to engineer functional single chain versions of an antibody with chorismate mutase activity, selecting for optimal linkers to join the relevant V_H and V_L domains. We have also established a sensitive growth selection assay in *E. coli* for catalysts with chorismate mutase activity, allowing for the direct selection for improved variants of our first-generation abzymes. Finally, we have utilized a sensitive immunoassay to screen antibody phage libraries directly for catalysis of a bimolecular Diels-Alder reaction and have identified several active clones. Detailed characterization of these molecules will allow the efficacy of standard hybridoma techniques and phage display methods to be directly compared.

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FINAL REPORT

GRANT #: N00014-90-J-4089 R & T CODE: 441s011 01

PRINCIPAL INVESTIGATOR: Donald Hilvert

INSTITUTION: The Scripps Research Institute

GRANT TITLE: Genetic selection for improved abzymes in *E. coli.*

AWARD PERIOD: 1 September 1990 - 31 August 1993

OBJECTIVE: To exploit molecular biological techniques to identify first-generation antibody catalysts and to improve their chemical efficiency.

ACCOMPLISHMENTS: Over the three-year course of this project we achieved three important goals: (1) we prepared functional single chain versions of the chorismate mutase antibody 1F7 using phage display technology; (2) we established a sensitive growth selection assay in *E. coli* for catalysts with chorismate mutase activity; and (3) we identified new abzymes for a Diels-Alder cycloaddition from a phage library.

(1) Single-chain antibodies. Efforts to prepare functional single-chain versions of the catalytic antibody 1F7 (sc1F7) by conventional means have proved unsuccessful. We consequently turned to phage display methods to select optimal linkers for connecting the heavy and light chain variable domains. The genes encoding the V_L and V_H portions of 1F7 were linked via a 48 bp random oligonucleotide, and the resulting construct was cloned into the phagemid vector pComb3, allowing the corresponding single-chain fragment to be displayed on the surface of the M13 phage virion as a fusion with phage coat protein III (cpIII). Functional antibodies were selected by multiple rounds of affinity chromatography with the transition state analog and amplification of the best binders. We have identified functional clones for both the V_L-linker-V_H and V_H-linker-V_L arrangements. In contrast to previously reported linkers which are mostly composed of small and uncharged residues, the sequences identified from the phage libraries contain a large number of prolines and charged amino acids.

Although we have yet to optimize the expression of *soluble* sc1F7, we successfully overproduced one of our clones (sc1F7-34) as inclusion bodies in *E. coli* using a T7 expression system. High expression efficiency required optimization of codon usage for three clustered arginines in CDR3 of the heavy chain. The appearance of three rare codons at these positions may account for our previous difficulties expressing 1F7 fragments in bacteria. Typical yields of antibody in the optimized system were 70-90 mg of inclusion bodies per liter of culture. The protein was subsequently refolded in 10-20% yield using standard methods. Sc1F7-34, like the parent antibody, catalyzes the rearrangement of chorismate into prephenate. Although it is less stable than the original IgG, the single-chain construct is somewhat more active (k_{cat} = 0.09 min^{-1} vs 0.03 min^{-1}). By increasing the stringency of the selection process and by evaluating larger numbers of clones for efficient expression, we hope to identify more robust agents. In any event, these studies illustrate the utility of phage display for engineering novel single-chain antibody molecules. Moreover, this methodology affords a general approach for identifying suitable peptide sequences for covalently tethering any two protein domains together in the absence of detailed structural information.

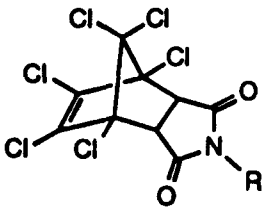
A-1

(2) Genetic selection. For genetic selection experiments with 1F7, we require a suitable host system featuring deficient chorismate mutase activity as the sole growth limitation. We have constructed *recA*⁻ *E. coli* strains KA11 and KA12 that have deletions of the genes encoding the two endogenous chorismate mutases (*pheA* and *tyrA*) and also lack the associated prephenate dehydrogenase (PDH) and prephenate dehydratase (PDT) activities. A complementation strategy based on the spontaneous conversion of prephenate to phenylpyruvate in acidic medium, as previously described by K. Backman (OmniGene), did not function appropriately under our conditions. Thorough analysis of the flow of relevant metabolites across bacterial membranes led to a new strategy based on resupplying the hosts with PDH and PDT. Genes encoding monofunctional forms of these enzymes (devoid of chorismate mutase activity) were obtained from R. Jensen (U. Florida). PDH activity levels produced by the *pheA-tyrA* deletion strains transformed with a plasmid carrying the *Pseudomonas aeruginosa pheC* gene were 10^2 times higher than the induced chromosomal level, while the PDT deficiency could be complemented with a plasmid bearing the *Erwinia herbicola tyrA* gene.

To allow for either a cytoplasmic or a periplasmic location of the chorismate mutase activity, two distinct versions of the complementation system were designed. Several plasmids containing the genes for cytoplasmic or periplasmic forms of the relevant biosynthetic enzymes PDH and PDT expressed either from the *lac* UV5 or the five-fold stronger *tac* promoter were constructed. These plasmids are compatible with plasmids carrying the chorismate mutase function, encoded either by genes for sc1F7 or the *B. subtilis* enzyme (*aroH*, obtained from J. Knowles, Harvard University). Results from *in vivo* tests with plasmidial *aroH* demonstrate that: *(i)* The cytoplasmic complementation system is fully operational as designed, with options to use both independent branches leading to Phe and/or Tyr for complementation of strains KA11 and KA12. Each of the three enzymes that were introduced from three different species perform in *E. coli* as predicted. *(ii)* Whereas production of PDH and PDT from a *lac* UV5 promoter is sufficient and well tolerated by the cells, the use of the stronger *tac* promoter causes severe growth impairment, either because the overproduced proteins themselves are toxic or because of metabolic imbalances caused by their activity within the cell. The possibility of fine-tuning expression levels by having different promoter options proved to be very effective. *(iii)* Three sets of conditions for varying stringency are available for selection. Selection for complementation of the Phe⁻ phenotype is the least stringent, since there already exists a considerable background growth due to Phe availability from an unknown source in the cell. This should allow for enrichment of rather weakly active chorismate mutase genes. The complementation of the Tyr auxotrophy appears to be much more demanding, since there was no discernible background growth observed in the absence of chorismate mutase. The most stringent option would be the complementation of both Phe and Tyr auxotrophies. Such a selection strategy promises to be the most successful in suppressing any background growth due to unrelated host or plasmid mutations. Experiments to create the periplasmic version of this complementation system are in progress, as are efforts to exploit these systems for characterizing and optimizing the activity of catalytic antibodies with chorismate mutase activity.

(3) Diels-Alderase antibodies from phage libraries. We have generated combinatorial antibody libraries from mRNA isolated from the spleens of mice hyperimmunized with transition state analogs for Diels-Alder and decarboxylation reactions (**1** and **2**, respectively). Numerous binders have been identified in both cases after multiple rounds of panning. We have been able to screen the library of binders to **1** directly for catalytic

activity using a recently devised immunoassay. The latter procedure (catELISA) involves the reaction of immobilized dienophile (an *N*-alkylmaleimide) with diene (tetrachlorothiophene dioxide) in solution and subsequent detection of the product with a monoclonal antibody as in a regular enzyme linked immunosorbent assay (ELISA). Several Fab-producing clones exhibit considerable activity in this assay and will be characterized to determine their catalytic efficiency relative to the previously reported antibody 1E9 which was prepared by standard hybridoma techniques.



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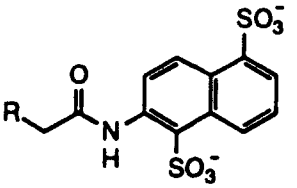

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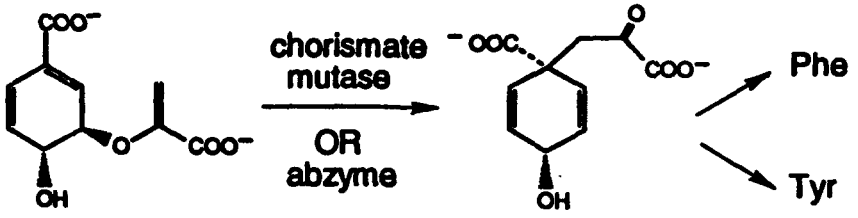
SIGNIFICANCE: Monoclonal antibodies with tailored catalytic activities and specificities can now be prepared on demand. However, the chemical efficiency of these catalysts is typically considerably lower than that of natural enzymes. Molecular genetics represents a potentially powerful tool for identifying first generation catalysts, redesigning their architecture, and optimizing their properties for potential practical applications in research, industry and medicine. The application of phage display methods and bacterial selection systems, as outlined above, promise to be important tools for achieving these overarching goals.

PUBLICATIONS: A publication describing the engineering of sc1F7 is in preparation. The work on the selection system for chorismate mutase activity and the selection of Diels-Alderase catalysts using phage display will be reported in due course.

GENETIC SELECTION FOR IMPROVED ABZYMES IN *E. COLI*

D. Hilvert, Research Institute of Scripps Clinic; 1993

Shikimate Pathway for Aromatic Amino Acid Biosynthesis



Objectives

- Express functional abzyme with chorismate mutase activity in *E. coli*
- Establish growth selection assay for abzyme activity
- Identify variants with improved catalytic efficiency following random mutagenesis and selection
- Use phage display methods to identify new abzymes

Accomplishments

- Constructed phage libraries of single-chain Fv proteins containing random linker sequences
- Isolated functional single-chain abzymes with higher chorismate mutase activity than parent IgG from these libraries
- Designed and evaluated *E. coli* complementation system for improving chorismate mutase abzymes
- Successfully screened combinatorial libraries by catELISA and identified catalytic antibodies for a bimolecular Diels-Alder reaction

Significance

- General method for creating highly efficient abzymes
- Establish structure-function relationships for protein catalysis
- Control cellular function, alter metabolism and destroy toxins with intracellular abzymes